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THE INVESTIGATION OF CYTOKINE ACTIVATION AND GENE-POLYMORPHISMS IN *HELICOBACTER PYLORI* INFECTION AND IN CROHN'S DISEASE

Ph.D. Thesis

by

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CONTENTS i

ABBREVIATIONS iii

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AP-1:	activating protein-1
ARMS:	amplification refractory mutation system
ATP:	adenosine triphosphate
BabA:	blood-group antigen-binding adhesin
CAG:	chronic atrophic gastritis
cagA:	citotoxin-associated gene A
CagA:	citotoxin-associated protein A - the product of the *cagA* gene
CARD:	caspase-recruitment domain protein
CD:	Crohn's disease
DC:	dendritic cell
DNA:	deoxyribonucleic acid
DU:	duodenal ulcer
EAE:	experimental autoimmune encephalomyelitis
ELISA:	enzyme linked immunosorbent assay
FODMAPs:	Fermentable Oligo-, Di- and Mono-saccharides And Polyols
GC:	gastric cancer
GITR:	glucocorticoid-induced TNF receptor-related protein
GU:	gastric ulcer
H. pylori:	*Helicobacter pylori*
HIV:	human immunodeficiency virus
HSP70-2:	heat shock protein 70-2
HWE:	Hardy-Weinberg equilibrium
IBD:	inflammatory bowel disease
IFN-γ:	interferon-γ
IgG:	immunoglobulin G
IL-1 RN:	IL-1 receptor antagonist
IL-10:	interleukin-10
IL-12:	interleukin-12
IL-1β:	interleukin-1β
IL-2:	interleukin-2
IL-6:	interleukin-6
IL-8:	interleukin-8
iNOS:	inducible nitric oxide synthase
LBP:	LPS binding protein
LPS:	lipopolysaccharide
MAP:	*Mycobacterium avium paratuberculosis*
MDP:	muramyl dipeptide
NAP:	neutrophil activating protein
NF-AT:	nuclear factor of activated T cells
NFκB:	nuclear factor κB.
NO:	nitrogen monoxide
NOD:	nucleotide-binding oligomerization domain
OMP:	outer membrane protein
PAI:	pathogenicity island
PCR:	polymerase chain reaction
PUD:	peptic ulcer disease
RFLP:	restriction fragment length polymorphism

RNA:	ribonucleic acid
RNI:	reactive nitrogen intermediates
ROI:	reactive oxigen intermediate
SabA:	sialic acid-binding adhesin
SCDID:	severe combined immunodeficient
SNP:	single nucleotide polymorphism
STAT:	signal transducer and activator of transcription
TGF-β:	transforming-growth factor-β
Th1:	T helper 1 cell
Th2:	T helper 2 cell
TLR4:	toll-like receptor 4
TLR5:	toll-like receptor 5
TNBS:	trinitrobenzene sulfonic acid
TNF-α:	tumor necrosis factor-α
Treg:	T regulatory cell
UC:	ulcerative colitis
vacA:	vacuolating cytotoxin gene A
VacA:	vacuolating cytotoxin A - the product of the *vacA* gene
WBC:	whole blood culture
WHO:	World Health Organisation

1. INTRODUCTION

Gastrointestinal inflammatory events associated with *Helicobacter pylori (H. pylori)* infection and inflammatory bowel disease (IBD), specifically with Crohn's disease (CD), are the result of a complex and fragile equilibrium between the pathogenic bacteria (*H. pylori* and commensal microflora, respectively) and their virulence machinery, on the one hand, and the cascade of inflammatory mediators released by the host's mucosa on the other. The host's immunological genetic background is as important as the virulence characteristics of the infecting bacteria in influencing the outcome of disease. In many gastrointestinal diseases research aiming at finding cures and not just symptom amelioration should approach therapeutic methods with a focus on both the bacterial and the host side of pathogenic processes.

In case of *H. pylori*-related diseases, research has yielded substantial results in therapies focusing on bacterial pathogenic factors. Antibiotics are able to eradicate *H. pylori* from the majority of the infected patients. Despite this commendable progress the total eradication of *H. pylori* remains elusive, mainly because *H. pylori* resistance against anti-microbials is increasing. Thus, new options in the armamentarium against *H. pylori* are necessary. One solution is the introduction of vaccines for therapeutic or prophylactic use. Promising advances have been made in this field with possible future implications. Other therapeutic interventions could be found by a better understanding of the pathogenic processes depending on host factors.

In case of Crohn's disease, novel therapies are unknown against the microbes involved in the pathogenesis, thus to address host pathogenic factors remains a straightforward option.

Therefore, in our experiments we focused on (1) the elements of the inflammatory process during *H. pylori* infection and on (2) host genetic factors presumably determining the cytokine response observed both in *H. pylori*-related diseases and Crohn's disease.

1.1. *HELICOBACTER PYLORI* INFECTION

Earlier, it was accepted that the stomach contains no bacteria and is actually sterile. However, in 1983, Marshall and Warren isolated *H. pylori* from clinical biopsies.[1] Marshall proved Koch's third postulate by drinking a turbid culture of a strain of *H. pylori* and thus acquiring gastritis with bacteria in the mucin layer over the inflamed lesion.[2] This provocative experiment launched revolutions in gastroenterology and microbiology. The significance of

Marshall's and Warren's work was acknowledged by the Nobel assembly, awarding the scientists a Nobel Prize in Medicine in 2005.[3] Further investigations showed that *H. pylori* is a Gram-negative, spiral shaped bacterium that lives in the stomach and the duodenum. It has a unique way of adapting to the harsh environment of the stomach. In spite of it's late discovery, it is estimated that *H. pylori* has infected humans since before the major migrations of *Homo sapiens* out of Africa 50 000-100 000 years ago.[4]

Epidemiologic studies show that the acquisition of *H. pylori* can lead to life-threatening diseases.[5] The disorder most unequivocably associated with *H. pylori* infection is peptic ulcer disease (PUD).[6] That infection with the bacteria is a key factor in the development of duodenal and gastric ulcers is proven by the fact that bacterial eradication results in the cure of existing ulcers.[7] A close correlation was demonstrated between acquisition of *H. pylori* and two other diseases: gastric MALT lymphoma[8] and gastric cancer (GC).[6,9] Underlying the importance of *H. pylori* as a first line causative agent in the induction of gastric tumours, the World Health Organization (WHO) classified the bacteria as a group I carcinogen for humans.[10]

Results of studies addressing the connection of *H. pylori* with extra-alimentary diseases are ambiguous. A positive association was reported between *H. pylori* infection and cardiovascular disease and atherosclerotic stroke.[11] Other investigations did not confirm these results.[12] Large scale epidemiologic trials also demonstrated that *H. pylori* infection may reduce the risk of atopic disorders: asthma, eczema and allergic rhinitis[13], while data show that *H. pylori* eradication should be included in chronic urticaria management.[11]

H. pylori infection is usually acquired in childhood and unless eradicated by treatment with antibiotics for other reasons, persists for the life of the patient. The prevalence among middle-aged adults is over 80 % in many developing countries[14], as compared with 20 to 50 % in industrialized countries.[15] More than 50 percent of the world population is infected[16], this is approximately true for Hungary as well.[17,18] It seems likely that in industrialized countries direct transmission from person to person by vomitus, saliva or feces predominates.[19] Opinions are contradictory as to additional transmission routes, such as water.[20] The person-to-person infection largely depends on socioeconomic and sanitary conditions.[21] In addition to these environmental circumstances, other factors - both bacterial and host- can impact on the persistence and long-term effects of *H. pylori* infection. From the bacterial side the pathogenic features of *H. pylori* strains greatly influence the outcome of the infection.[22] Whereas, from the host side, the genetic background determines the susceptibility to *H. pylori* and its maintenance.[23]

Despite the numerous people infected by H. pylori, data show that most patients have no outward symptoms, and "only" 10-20% of individuals carrying the bacteria are threatened by the serious problems listed above.[24] Thus, in our studies we focused on investigating bacterial and host factors underlying the susceptibility to H. pylori infection.

1.1.1. Pathogenic factors of *H. pylori*

The gastric mucosa is well protected against bacterial infections by variable acidity, by rapidly changing concentrations of nutrients and by a host response leading to chronic inflammation and activation of a panel of immune defense mechanisms. *H. pylori,* however, is able to adapt to this ecologic niche, with a unique array of features that permit entry into the mucus, attachment to epithelial cells, evasion of the immune response and as a result persistent colonization.[25] In addition, the crosstalk between bacterium and host maintains a fine tuned balance between inflammation enhancement and anti-inflammatory effects. In this delicate balance virulence factors such as the *cag*-asssociated type IV secretion system and the vacuolating toxin (VacA) are of critical importance.[26] The *H. pylori* genome (1.65 million bp) codes for about 1500 proteins.[27] The most remarkable findings of *H. pylori* genome sequencing projects were the discovery of outer membrane proteins (OMPs) that include most known *H. pylori* adhesins and the discovery of many genes that can be switched on and off by slipped-strand mispairing-mediated mutagenesis.[28] These genes are responsible for encoding enzymes that modify the antigenic structure of surface molecules and control the entry of foreign DNA into the bacteria. *H. pylori* is a particularly extreme example of bacteria with both a high mutation rate and a very high recombinational frequency.[29,30] The genome of *H. pylori* changes continously during chronic colonisation of an individual host by importing small pieces of foreign DNA from other *H. pylori* strains during persistent or transient mixed infections.[31] Thus, each host is colonized rather by a cloud of usually closely related organisms, resembling the "quasispecies" observed with persistent RNA viruses such as hepatitis C and HIV.[32]

1.1.1.1. Urease

The first barrier that the bacterium must overcome is the low pH in the lumen of the stomach. To do this, *H. pylori* produces urease which utilizes nickel as a cofactor. Urease mediates acid resistance via hydrolysing urea into carbon dioxide and ammonia, which effectively buffer the surrounding microenviroment and bacterial cytosol.[33] The enzyme

activity is regulated by a unique pH-gated urea channel UreI that is open at low pH and shuts down the urea-influx under neutral conditions.[34]

1.1.1.2. Flagella and adhesins

Motility is essential for colonization.[26] *H. pylori* possesses five or six polar *flagella* consisting of two structural subunits, referred to as FlaA and FlaB.[35] After swimming through the viscous mucin layer, approximately 20% of *H. pylori* bind to gastric epithelial cells with help of proteins belonging to the OMP group, the *adhesins*. The most important ones are the sialic acid-binding adhesin (SabA), the blood-group antigen-binding adhesin (BabA), the HopQ and a virulence-associated OMP encoded by *oipA*.[36] *H. pylori* strains positive for these OMPs are associated with an increased risk for glandular atrophy, intestinal metaplasia and enhanced epithelial cell proliferation, which may explain the augmentation in GC risk.[37] OMPs encoded by *oipA* carry the strongest bacterial risk factor for high colonization, intense gastritis, increased mucosal interleukin-8 (IL-8) levels and duodenal ulcer (DU) disease within patients with varying disease outcomes.[38]

1.1.1.3. The *cag* pathogenicity island (*cag* PAI)

Most strains of *H. pylori* possess the *cag* PAI which now has been recognized as a marker for strains that confer increased risk for peptic ulcer disease[39] and GC.[40] This island is a 35-40-kb genomic fragment containing 31 genes.[25] These genes encode components of a type IV. secretion apparatus that translocates the 120-kD protein CagA into the host cell.[41] This type IV. secretion system can be found in other bacteria as well and is responsible for injecting macromolecules (i.e. DNA and proteins such as pertussis toxin) into host cells.[42] However, no homologues are known for *cagA* in other *Helicobacter* species or in other bacteria, suggesting that it reflects a human gastric specific gene.[43] After entering the epithelial cell, CagA is phosphorylated and binds to SHP-2 tyrosine phosphatase, which affects spreading, migration, and adhesion of epithelial cells. This phenomenon can be assessed *in vitro* by a change in epithelial cell morphology to the scattered, or "hummingbird" phenotype.[44] The CagA protein has also been implicated in disruption of the apical-epithelial junction.[45] Another important function of the *cag* PAI is the induction of a growth factor-like cellular response and intense chemokine secretion by the host cell, such as IL-8.[46]

1.1.1.4. Vacuolating cytotoxin A (VacA)

Factors involved in disease progression include the VacA, a secreted exotoxin. The 95-kD protein causes massive vacuolar degradation of epithelial cells *in vitro* and epithelial erosion *in vivo*.[47] VacA is encoded by the *vacA* gene which comprises two variable parts.[48] The *s*-region (encoding the signal peptide) is located at the 5' end of the gene and exists as an *s1* and *s2* allele. The *m*-region (encodes part of the toxin cell binding domain) occurs as an *m1* or *m2* allele. The mosaic combination of *s*- and *m*-region allelic types determines the production of the cytotoxin and is thereby associated with pathogenicity of the bacterium.[48] *vacA s1/m1* strains are most closely associated with gastric carcinoma.[49] Unlike *cagA*, v*acA* is conserved among all *H. pylori* strains, although significant polymorphism exists.[48] The *cag* island and *vacA* are far apart on the *H. pylori* chromosome, yet there is a strong statistical linkage between the *s1* genotype of *vacA* and the presence of the *cag* island.[48] VacA forms channels in the plasma membrane of target cells, which enable the release of important nutrients, such as bicarbonate, pyruvate and urea.[50] In addition VacA causes the loosening of tight junctions, resulting in transport of Fe^{3+}, Ni^{2+} ions and larger molecules, such as mannitol and sucrose, to the nutrient-poor region under the mucous layer.[51] This might also enable the transepithelial crossing of other virulence factors to their underlying cellular targets.

1.1.1.5. Neutrophil activating protein (NAP)

Another virulence factors is NAP, which is a cytosolic protein, released by bacterial lysis, interacting directly with neutrophils, monocytes and mast cells, resulting in the activation of their inflammatory functions. The function of NAP in aggravating the innate immune response, especially the neutrophil attack might be to obtain nutrients from the inflamed tissue. NAP forms a dodecamer with sequence and structural charactersistics related to iron-binding proteins.[47]

1.1.2. Host response to *Helicobacter pylori*

H. pylori infection induces inflammation of the gastric mucosa. This inflammatory response consists of the recruitment of neutrophils, monocytes and macrophages with consequent production of reactive oxigen and nitrogen intermediates (ROIs, RNIs), pro-inflammatory cytokines, activation of inducible nitric oxid synthase (iNOS), and histamine release. This is followed by the activation of the adaptive immune system with T and B lymphocyte and plasma cell recruitment, antibody production and epithelial cell-damage. *H. pylori* triggers and maintains gastric mucosal inflammation by different

mechanisms, which depend greatly on the virulence of the infecting strain, being much higher when infections are caused by *cag A* and *vac A* positive strains, although the main strain-associated inducer might be *cag A* associated functional *oip A*.[52]

In spite of the rapid and effective onslaught of the innate and adaptive immune response, *H. pylori* is able to persist for decades in infected patients with various strategies to evade host immunity. This lifelong colonization, however, many times depends on individual host characteristics as well.

1.1.2.1. Innate immune response I.: Oxidative stress, iNOS activation, histamine release, phagocytosis

The release of NAP, urease and porins by *H. pylori,* the damage of epithelial cells and furthermost the activation of the potent neutrophil chemotactic factor, IL-8 recruits neutrophils, monocytes and mast cells to the site of invasion (Fig. 1.). The production of *ROIs, RNIs* is induced to destroy the bacteria through the activation of NADPH oxidase. To avoid the negative effects of ROIs, *H. pylori* produces enzymes involved in scavenging, such as catalase and superoxide dismutase and NADPH–dependent quinine reductase.[53]

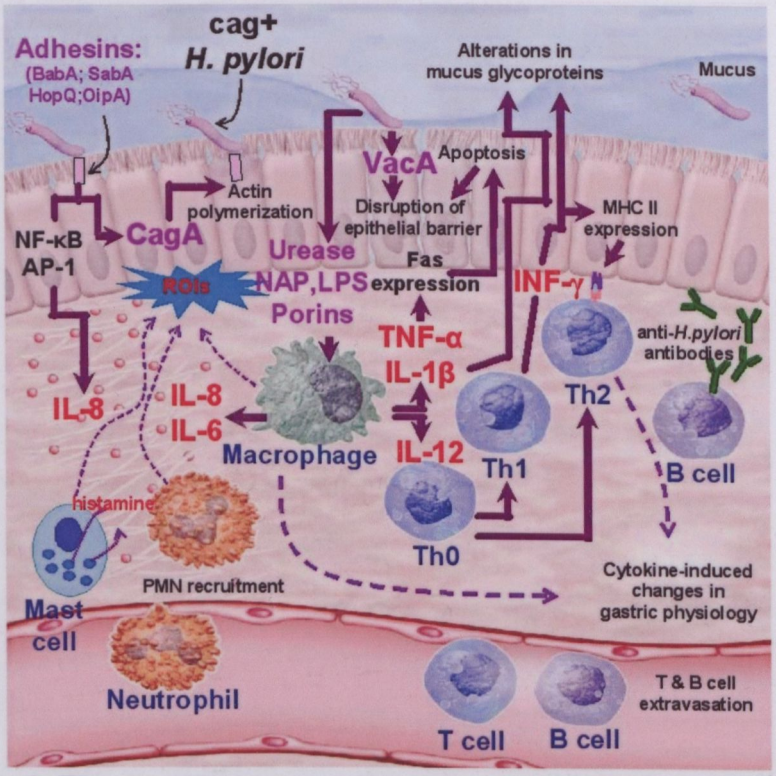
H. pylori infection can lead to increased *iNOS production* by activated macrophages and neutrophils in the gastric mucosa. This is also associated with epithelial cell damage and apoptosis. In macrophages, the bacterial urease has been implicated in iNOS activation.[54] To avoid killing by NO, *H. pylori* produces an arginase which converts L-arginine to urea and L-ornithine.[55] Because L-arginine is also used by iNOS to produce NO, arginase can compete with iNOS for their common substrate and regulate NO synthesis.[56] In the presence of oxygen free radicals, NO can form a genotoxic peroxynitrite, which can contribute to further destruction of the mucosa. Nitrotyrosine as a stable end product of peroxynitrite, can be used for the detection of NO and peroxynitrite formation.[57]

H. pylori infection elicits a complex immune response which also leads to the activation of mucosal mast cells[58], the infiltration of basophil granulocytes[59] and consequent mucosal *histamine release*. Recently basophils were shown to exhibit chemotaxis in response to the *H. pylori*-derived peptide Hp(2-20).[59] It was shown that *H. pylori* stimulates histamine release and DNA synthesis in rat enterochromaffin-like cells as well.[60] Histamine modulates cytokine production via the H_1 and H_4 receptors besides affecting gastric acid secretion (H_2 receptors) and neurotransmitter release (H_3 receptors). Thus, the inflammatory process is further enhanced.

Several studies indicate that, although effectively ingested by professional *phagocytes, H. pylori* is more resistant to killing than other Gram-negative bacteria. In vitro studies demonstrate that phagocytosis of *H. pylori* by macrophages is delayed and that viable bacteria accumulate in larger than normal phagosomes, reffered to as megasomes, which result from homotypic fusion.[61] Subsequent macrophage apoptosis might enable the escape of bacteria.[62] Additionally, *H. pylori* has evolved in such a way that bacterial components that are normally recognized by the host innate immune system are not recognized. One example is the lipopolysaccharide (LPS), which in *H. pylori* is considerably less reactogenic.[61] The other is the *H. pylori* flagellin, which is poorly recognized by the Toll-like receptor 5 (TLR5).

1.1.2.2. Innate immune response II.: Pro- and anti-inflammatory cytokine production

The role of cytokines in the initiation and modulation of gastrointestinal inflammatory response is crucial. The gastric epithelium of *H. pylori*-infected individuals can be characterized by enhanced levels of pro-inflammatory cytokines. High mucosal levels of mononuclear -IL-8[63], IL-6[64], IL-1β[65], TNF-α[66] and interferon gamma (IFN-γ)[67] have been described in *H. pylori* infected patients (Fig. 1.). Lymphocytic derived cytokines -IL-2-[68] have been also detected. Among these, IL-8, a potent neutrophil-activating chemokine, secreted by epithelial cells apparently has a central role.[69] It is LPS, urease and other strain-related virulence products (encoded by *oipA, cagA*), which induce the secretion of IL-1β, TNF-α, etc. from monocytes and dendritic cells, thus up-regulating the expression of chemokines from epithelial cells, such as IL-8. *H. pylori* like many enteritis-inducing intestinal pathogens will also directly stimulate epithelial chemokine secretion. *H. pylori* strains carrying the *cag* PAI induce a far stronger IL-8 response than *cag*-negative strains, and this response depends on the activation of nuclear-



1. Fig. Pathogen – host interactions in the pathogenesis of *H. pylori* infection. (PMN: polymorphonuclear cell, other abbreviations are listed in the text)

factor-κB (NF-κB) and the early–response transcription factor activating protein-1 (AP-1).[70] It has been demonstrated that *H. pylori* also induces the production of IL-12, a heterodimeric pro-inflammatory protein that is secreted by dendritic cells and phagocytes in the gastric mucosa.[71] IL-12 further induces the production of IFN-γ.[71]

In chronic infections, such as persistent *H. pylori* infection the activation of chemokine cascades and cell activation would likely to be detrimental. The host has evolved ways of inhibiting pro-inflammatory responses. In *H. pylori* infection increased anti-inflammatory cytokine production, in the form of IL-10 has been identified as a protective mechanism. In *H. pylori* infected human gastric tissue an elevated level of the anti-inflammatory cytokine IL-10 was detected by immunohistochemistry. It has recently been suggested that the subclass of T lymphocytes, T regulatory cells (Tregs), may be responsible by inducing production of IL-10, thus reducing the grade of inflammation and influencing the outcome of *H. pylori* infection.[72]

1.1.2.3. Host genetic background-cytokine gene polymorphisms

The host genetic background also has a strong influence in determining the susceptibility to *H. pylori* infection and its maintenance. A twin study reported that the concordance of anti-*H. pylori* antibody status was higher in monozygotic than in dizygotic pairs which strongly indicates a genetic role.[73] As mentioned above, the local inflammation in *H. pylori* infection is characterized by increased production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α. Such cytokine production and consequently the pattern and severity of inflammation are often determined by promoter and structural polymorphisms of regions involved in cytokine gene expression. Polymorphisms are DNA sequence variations, which are frequent in regions of the gene that regulate transcription or post-transcriptional events. If the frequency of these variations is higher than 1% in a given population, then they are referred to as polymorphisms. If the frequency of these variations is lower than 1%, then they are considered mutations. Additionally, if the DNA sequence variation is a one nucleotide change, than it is called a single nucleotide polymorphism (SNP). Cytokine gene polymorphisms in connection with *H. pylori* infection are not discussed in their entirety below. However, those cytokine gene polymorphisms are described in detail, which have been most extensively investigated in the literature or in our experiments.

IL-1: In *H. pylori* infection IL-1β (-511 C→ T) and IL–1 receptor antagonist allele 2 (IL-1 RN 2) (2 identical tandem repeats of 86 bp in length, in intron 2) gene polymorphisms play a pivotal role in modulating the inflammatory response.[74] The polymorphism of IL-1β

gene favors the development of gastritis predominantly in the body of the stomach that is associated with hypochlorhydria, gastric atrophy and gastric adenocarcinoma.[74] In the absence of this polymorphism, *H. pylori* mediated gastritis develops in the antrum in association with normal to high level of acid secretion.[74] Other studies give further support to the fact that in patients with peptic ulcer and gastric adenocarcinoma heterozygous IL-1RN 1/2 and IL-1β C/T genotypes are more frequent than in patients with gastritis.[75] In addition, when the influence of CagA and VacA status on histological changes was analyzed in subjects stratified according to IL-1β and IL-1 RN polymorphisms, it appeared that more severe gastric abnormalities correlated both with the high risk polymorphisms (IL-1*β* -511 T; IL-1RN 2) and virulence factors (*cagA+/vacA s1/m1*). Thus, a synergistic effect of bacterial and host factors could be observed.[76]

TNF-α: Polymorphisms in the TNF-α gene have been tentatively associated with an increased risk of inflammation and more severe disorders. The first reports regarding this issue described an association between the TNF-α -308 G/G genotype and the risk of *H. pylori*-associated duodenal ulcer.[77] More recent findings showed an association between the TNF-α (-308 G→ A) SNP and GC, chronic atrophic gastritis (CAG), intestinal metaplasia, and gastric ulcer (GU) both in the Portuguese and the Korean population.[78,79] This SNP is associated with an increased production of TNF-α..[80] The analysis of the combined IL-1β (-511 T carriers), IL-1 RN (IL-1 RN 2/2) and TNF-α (-308 A carriers) genotypes showed that the joint effect of these polymorphisms exacerbated the risk for both CAG and GC development.[79] Among other TNF-α SNPs, the TNF-α (-857 C→ T) polymorphism was correlated with DU, while the TNF-α (-1031 C→ T) SNP was associated with increased antral inflammation.[81] Whereas, an interesting finding is that the TNF-α -238 A allele provides protection against GC.[82]

IFN-γ: IFN-γ, produced by NK cells, Th1 cells, CD8+ cytotoxic lymphocytes and macrophages, is also suggested to play a significant role in the pathogenesis of *H. pylori*-associated inflammation. A variable length CA repeat polymorphism in the first intron of IFN-γ gene has been found to be associated with IFN-γ production and the high producer allele 2 was in complete linkage with the T allele of a second polymorphism of intron 1 (+874 T→ A).[83] Additionally, an association was shown between an SNP (-56 C→ T) in the promoter of the IFNGR1 gene, encoding chain 1 of the IFN-γ receptor and high anti-*H. pylori* antibody titer.[84]

IL-10: Several studies address the role of IL-10 SNPs in the development of gastric malignancies. El Omar et al. described an association between the low IL-10 producing haplotype, which comprised the IL10 -819 T allele, and the risk of non-cardia gastric cancer.[85] It has already been suggested that this allele is connected with mild *H. pylori*-associated inflammation and with persistent infection, possibly due to the low amount of IL-10 produced in response to the infection itself.[81] In addition, in another study the IL-10 (-819 C→ T) SNP was significantly correlated with the risk of non-cardia gastric cancer and with precancerous intestinal metaplasia.[81]

IL-8: Some interleukin polymorphisms may influence the ability of *H. pylori* to persist in the gastric mucosa.[81,85] In a large group of Japanese patients, Hamajima et al. showed that the risk of seropositivity used as marker of continuous infection is increased in subjects harbouring a combination of IL-8 and IL-10 polymorphisms associated with mild inflammation. This combination associates IL-8 (-251 T/T genotype) with IL-10 (-819 T/T genotype).[86]

1.1.2.4. Adaptive immune response- Th1 dominance

Evidence from both human[87] and murine studies[88] suggest that the T-helper response in the gastric mucosa in *H. pylori* infection is predominantly associated with a Th1 profile characterized by IFN-γ and TNF-α, but not IL-4 or IL-5 -secreting effector cells. Th1 cells are induced mostly in response to intracellular pathogens, whereas Th2 cells stimulate B cells in response to extracellular pathogens. Because *H. pylori* is non-invasive and induces a strong humoral response, a Th2-cell response would be expected. Paradoxically, *H. pylori*-specific gastric mucosal T cells generally present a Th1 genotype.[89] Studies in gene-targeted mice have further showed that Th1 cytokines promote gastritis, whereas Th2 cytokines are protective against gastric inflammation.[90] In fact, gastric inflammation and atrophic changes are abrogated in the absence of the key Th1 cytokine IFN-γ and are induced by IFN-γ infusion causing an increase in gastrin secretion and a decrease in somatostatin levels, even without *H. pylori*.[25] In vivo neutralization of IFN-γ by administration of specific antibodies also significantly reduced gastric inflammation.[25] High levels of IL-12, a Th1-stimulating cytokine, may contribute to the polarization of the T-cell response.[87] IL-12 is a potent stimulator of NK cells as well, and these cells will secrete IFN-γ following stimulation by IL-12. Both IFN-γ and IL-12 will polarize the differentiation of naïve T cells to cells with a Th1 profile. The IL-12 response observed following *H. pylori* infection, however, may be important in controlling the infection, as higher colonization levels were observed in IL-12

knockout mice with wild-type and colonization-impaired mutants of *H. pylori*.[91] Studies also suggested that the cytokine profile of antigen-specific T cells may differ in patients with peptic ulcer disease compared to those with only chronic gastritis. In patients with ulcer disease the *H. pylori*-specific T-cell response was more polarized to a Th1 profile than in chronic gastritis.[92]

In spite of the overwhelming Th1 response, *H. pylori* infection induces a vigorous systemic and mucosal humoral response as well.[93] This immunoglobulin production and the associated complement activation does not lead to eradication of the infection but may contribute to tissue damage. There is increasing evidence that autoantibodies may be stimulated by *H. pylori* infection.[94,95] Some *H. pylori* infected patients have an autoantibody response directed against the H^+/K^+-ATPase of gastric parietal cells that correlates with increased atrophy of the corpus.[95]

1.2. CROHN'S DISEASE

Crohn's disease and ulcerative colitis (UC) are together referred to as inflammatory bowel disease. They lead to long-term and sometimes irreversible impairment of gastrointestinal structure and function. It was Burrill B. Crohn who reported a mysterious small intestinal disease in 1932, probably of infectious origin, which is now known as Crohn's disease.[96] Although CD and UC share many clinical and pathological characteristics, they also have some markedly different features, and there is now ample reason to believe that the main pathological processes in these two diseases are distinct. CD is a heterogeneous disorder with different clinical phenotypes. Any part of the gastrointestinal tract can be affected, but most commonly, the terminal ileum, cecum, peri-anal area and colon. It is characterized by the presence of segments of normal bowel between affected regions known as "skip" lesions. The intersection of linear ulcers with islands of normal or oedematous mucosa might produce a "cobblestone" appearance. Regarding the histology, a transmural, dense infiltration of lymphocytes and macrophages can be observed. The presence of granulomas in up to 60 % of patients, fissuring ulcerations and submucosal fibrosis are characteristic. The narrowing of the gut lumen can lead to strictures, bowel obstruction, abscess formation and fistulization to skin and internal organs. These complications frequently need surgical intervention. Extra-intestinal inflammatory manifestations in joints, eyes, skin, mouth and liver can occure. The disease's course varies widely, with periods of remission and exacerbation including abdominal pain, diarrhea and fatigue. Medical

treatments ameliorate symptoms but despite improved understanding of the etiology and pathogenesis of the disease, a cure remains elusive.

Both CD and UC primarily are diseases of young adulthood, with peak incidence occurring between 15 and 30 years of age. Epidemiologic studies show that IBD incidence and prevalence vary significantly depending on geographic location and racial or ethnic background. An increase can be detected in the incidence of CD over the past three decades in almost all Western countries. Some regions have leveled off at a high incidence per 100.000 at 6 (USA)[97], and 3.4 (Italy)[98], whereas others show continued increases at 4.1 (Denmark)[99], 5.9 (UK)[100] and 14.6 (Canada).[101] In Hungary, a recent report from Lakatos et al. reported an increase of both CD and UC in a province of Western Hungary from 1977 to 2001. In CD the incidence increased from 0.41 to 4.68 per 100.000 persons which is comparable to Western European countries.[102] In general, there is an increased risk for developing IBD in urban compared with rural areas, in cohorts with a higher socioeconomic class, as well as in developed rather than less developed countries.[103] Incidence has been found to increase when populations emigrate from low-risk geographic areas to those with higher risk.[104] Prevalence rates for IBD among non-Caucasians in the US have been reported consistently as being lower. However, follow-up prospective studies suggest that incidence and prevalence rates among African Americans are higher than reported previously.[105] This would be consistent with a cohort effect resulting from changing environmental exposures over time. Interestingly in Hungary ethnic differences can also be observed between the Roma (Gipsy) minority and the Caucasian population. Concentrated in some Hungarian regions, the incidence of IBD within the Roma minority is half compared to the rest of the population.[106]

1.2.1. Pathogenesis of CD

At present IBD is thought of as a multifactorial disease, which results from an inappropriate and exaggerated mucosal immune response to normal constituents of the mucosal microflora that is in part determined by environmental and genetic factors.[107,108]

1.2.1.1. Effector and Regulatory T cell abnormalities

The inflammation observed is dominated by an excessive *Th1-mediated effector cell response.* T cell priming requires the activation of dendritic cells (DC). Although the exact stimulus is unknown, various *in situ* immunohistological studies indicate that dendritic cells in CD overproduce the Th1 polarizing cytokine IL-12.[109] Macrophages isolated from the inflammatory lesions of patients with CD also produce increased amounts of IL-12 *ex vivo.*[109]

In addition, nuclear extracts of T cells from the affected tissues of patients with CD contain increased amounts of activated STAT4 and the transcription factor T-bet, which is indicative of IL-12 signalling.[110] T cells isolated from these tissues express increased amounts of the IL-12Rβ2 chain, which is further proof for Th1 cell involvement.[111] Therefore, it is not surprising, that T cells isolated from the affected tissues of patients with CD produce markedly increased amounts of IFN-γ together with decreased amounts of IL-4, compared with controls.[112] Further, definitive support has been obtained from the finding that in most patients treated with antibody specific for the p40 chain of IL-12 a prompt and marked amelioration of inflammation can be observed. Clinical improvement is associated with reduction in both the production of IL-12 and IFN-γ by mononuclear cells.[113] The IFN-γ produced by Th1 cells affect downstream effector cells, such as macrophages to induce further production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, which are integral to the pathological response and to disease initiation and propagation.[114] Increases in the levels of the putative initiating cytokines (e.g. IFN-γ) are relatively modest, about 3-fold, however increases in the levels of downstream pro-inflammatory cytokines are more substantial, about 10-20-fold.[115] This argues for the occurrence of a multiplier effect in IBD inflammation. The marked increases detected in the level of the aforementioned cytokines focused attention on ways of controlling the Th1 cell response by inhibition of the function of these cytokines. The use of specific antibodies gained ground. Clinical trials have found infliximab, a chimeric monoclonal immunoglobulin-G 1 (IgG1) antibody that binds and neutralizes soluble and membrane-bound TNF-α, to be effective in inducing and maintaining the remission of Crohn's disease in 60% of patients.[116,117] The success rate of infliximab is probably due, not to the fact that it blocks TNF activity, but rather to the fact that it induces apoptosis of Th1 effector cells, which presumably express cell-surface TNF.[118] Accordingly, the IL-6R-specific antibody[119], tested in experimental colitis, seems to function through this mechanism as well.

Anti-inflammatory processes are also disrupted. Relative deficiencies in IL-10 have been found.[120] Intestinal inflammation, chronic enterocolitis occurs in models in which there is a clear deficiency in the production or function of a known regulatory cytokine, such as the IL-10 knockout mice[121] or mice with defective transforming-growth factor-β (TGF-β) signaling.[122] Additionally, the elevated production of pro-inflammatory cytokines in CD biopsies can be down-regulated by exogenous addition of IL-10 *in vitro*.[123] On the other hand therapeutic trials in which recombinant human IL-10 was administered to CD patients yielded only modest results.[124]

Mouse models point to deficient *regulatory T cell responses* as well, in connection with CD pathogenesis. A notable example is mucosal inflammation that is induced in a severe combined immunodeficient (SCID) mouse by the adoptive transfer of naïve ($CD45RB^{hi}$) T cells lacking regulatory cells, which is prevented by the co-transfer of mature ($CD45RB^{lo}$) cells that contain the missing regulatory T cell subpopulation.[125] In this model the $CD25^+$, $CD4^+$ and glucocorticoid-induced TNF receptor-related protein $(GITR)^+$ suppressor regulatory cell subpopulation was used.[125] Colitis also occurs in mice (Tgϵ26) in which the abovementioned regulatory T cells fail to develop properly because of aberrant thymic microenvironment.[126] In other models there is a clear deficiency in the function of T_R1 regulatory cells resulting in lower levels of IL-10 and TGF-β.[121, 122] Finally a third type of T regulatory cell subpopulation ($CD8^+$) is shown to decrease in patients with IBD as a result of dysregulated expression of gp180, an antigen expressed by epithelial cells.[127]

1.2.1.2. Environmental factors and microbial flora

Susceptibility to the development of CD involves the harmful effect of environmental factors and the intestinal microbial flora. Incidence for CD is greater in urbanized, more industrialized areas and lesser in the rural countryside, as discussed above.[103] In addition, over a relatively short period of time an accelerated growth has been observed in the number of patients with CD, which is consistent with an environmental influence. The association of CD with westernization has implicated lifestyle factors in the pathogenesis. One of the likely candidates is the specific kind of "Western diet" that is characteristic of more developed countries. A new hypothesis has recently been proposed, by which excessive delivery of highly fermentable but poorly absorbed short-chain carbohydrates and polyols (designated FODMAPs) to the distal small intestinal and colonic lumen is a dietary factor underlying susceptibility to CD.[128] The subsequent rapid fermentation of FODMAPs in the distal small and proximal large intestine induces conditions in the bowel that lead to increased permeability, which is a predisposing factor to the development of CD.[128]

It is accepted universally that experimental colitis does not develop, when mice are kept in a germ-free environment and can be prevented and treated by broad-spectrum antibiotics.[107] The normal mucosal flora is required to initiate and maintain the inflammatory process.[107] Although several candidates have been proposed, the precise pathogenic bacterium is unknown. The most enduring infectious candidate has been *Mycobacterium avium paratuberculosis* (MAP). MAP has been detected in tissues of patients with CD by culture and molecular methods.[129, 130]

Another concept, which emerged parallel with other theories concerning the pathogenesis of CD, has been termed "dsybiosis". This concept suggests a breakdown in the balance between putative species of "protective" versus "harmful" intestinal bacteria in the pathogenic process in CD. Some bacteria are thought to exert an inflammatory effect, while others a protective role in IBD. The strain *Lactobacillus* subspecies *reuteri* was shown to reduce mucosal permeability, prevent the onset of colitis, and attenuate established inflammation in IL-10 -/- mice.[131] Contradictory results demonstrated that different *Lactobacillus* subspecies activate human dendritic cells, skewing T cells toward Th1 polarization, thus potentiating the immune response.[132] Strains of *Bacteroides* and *Clostridia* species can produce enterotoxins and/or possess proteolytic properties that enhance mucosal permeability and bacterial uptake.[133] Studies have also shown that adherent and invasive *E. coli* can often be found in ileal lesions of CD by colonizing the intestinal mucosa, crossing the epithelial barrier and inducing an immune response.[134]

1.2.1.3. Genetic factors

Epidemiological and family studies have provided overwhelming evidence that genetic factors have an important role in determining susceptibility to IBD.[105] In population-based studies 5 %-10 % of all affected individuals with IBD report a positive family history.[135] The most compelling evidence comes from studies that are conducted with twins. The rate of concordance for CD has been reported to be as high as 58 % in identical twins, whereas the dizygotic-twin concordance is not significantly different from that for all siblings.[136] These observations support the assumption that susceptibility to IBD, in particular CD, is inherited, but not as a Mendelian trait. On the contrary, it is thought that IBD has a complex genetic basis with many contributing genes. In the past years considerable progress has been made to implicate genetic factors as vital components in CD pathogenesis. Genome-wide screening and candidate gene analysis have revealed several genetic loci (IBD1-IBD8) that show significant association with CD.[105, 137] The 2 genetic loci that are most strongly related to CD are the IBD1 locus in the pericentromeric region of chromosome 16 and the IBD5 locus on chromosome 5.[105]

As in the case of the gene polymorphisms connected with *H. pylori* infection, only those polymorphisms are described below in association with CD, which have been most researched by others or have been investigated in our laboratory.

NOD2: Precise analysis of the IBD1 region identified a strong association with a single gene, the nucleotide-binding oligomerization domain 2 (NOD2), also known as caspase-recruitment domain protein 15 (CARD15).[137] Amongst the genetic variations observed in all loci connected with CD pathogenesis, the polymorphisms of the NOD2 gene are the best-characterized and have been investigated most extensively. NOD proteins are intracellular pattern-recognition receptors, of which NOD2 senses bacterial muramyl dipeptide (MDP) (Fig. 2.). NOD2 is involved in the nuclear factor κB (NFκB) signaling cascade, but the detailed mechanisms remain unclear.[105] It is expressed constitutively in myeloid cells, particularly macrophages, neutrophils and dendritic cells, as well as Paneth cells in the small intestine, which are of epithelial origin.[138] Several NOD2 polymorphisms have been identified, probably the most important are two missense mutations (2722 G→C and 2104 C→T) and one frameshift mutation, where a cytosine insertion at position 3020 in exon 11 (3020insC) can be found.[139, 140] Individuals with one of the three major disease-associated alleles have a 2-to 4-fold increased risk of developing CD, whereas homozygous or compound heterozygous carriers have a 15-to 40-fold increase in risk.[138] Despite their strong association with CD, mutated NOD2 alleles are neither sufficient nor necessary for the development of CD, since they occur in healthy individuals, with estimates most commonly around 0.5 %-2 % in the general population, and 60 %-70 % of CD patients show no NOD2 mutations.[138] Moreover disease penetrance is relatively low, less than 10 % of all NOD2 homozygotes are likely to develop CD.[105] It is probable that other genetic determinants are required for disease expression.

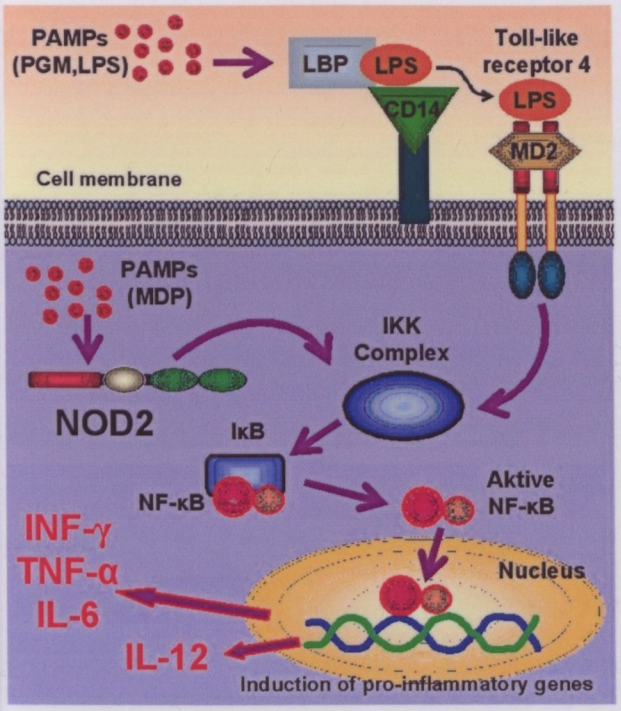


Fig. 2. Extracellular and intracellular signalling pathways of microbial-host interactions.
(IKK: κB kinase, IκB: inhibitory binding proteins κB, other abbreviations are listed in the text)

CD14: Besides the NOD2/CARD15 protein, another important pattern-recognition receptor, which mainly recognizes bacterial LPS is CD14, localized on chromosome 5. This receptor was also found to be involved in IBD pathogenesis.[141] Bacterial LPS are bound with high affinity to the LPS binding protein (LBP), circulating in the serum. The LPS/LBP

complex interacts with the LPS receptor CD14. CD14 is primarily expressed on the surface of monocytes, macrophages and activated neutrophils. The soluble form of this receptor (sCD14) is also found in the serum. The signal transduction of the LPS/LBP/CD14 ternary complex is mediated by the toll-like receptor 4 (TLR4) and results in activation of NFκB and production of pro-inflammatory cytokines such as IL-1, IL-6 and INFγ, similarly to the effect of the NOD2/CARD15 activated pathway (Fig. 2.).[141] An SNP in the promoter of the CD14 gene (-159 C→ T) has been described (often referred to as -260 C→ T by a few investigators due to numbering from the translation start) and in a few studies it was found to be functionally relevant in predisposing to CD.[141,142] In other reports the -159 C→ T CD14 SNP was shown to induce elevations in the amounts of both the membrane-bound[143] and the soluble form of the LPS receptor.[144] Interestingly, these results correlated with *H. pylori* infection induced sCD14 elevations.[144] In addition, in two recent investigations the co-existence of a mutation in either the CD14 or the TLR4 gene and in the NOD2/CARD15 gene increased susceptibility to developing CD compared to controls and patients with UC.[142,145] However, in spite of the abovementioned studies, a few research groups reported contradictory results with finding no association between CD14 gene variants and CD in the Japanese, Scottish and Irish populations.[146,147]

The activation of the microbial sensors, NOD2 and CD14 lead to the increased production of pro-inflammatory cytokines, which have a central function in disease propagation. Several studies have shown that genes encoding immunoregulatory cytokines are also important candidate susceptibility loci (e.g. IBD5) for IBD. Cytokine genes can influence CD disease phenotype.[148] As it has been determined, allelic variants of cytokine genes are associated with the amount of cytokine produced in both *in vitro* and *in vivo* models.[149-151]

TNF-α: Several cytokine polymorphisms, especially SNPs have been described in connection with CD and they were proven to regulate the amount of cytokine produced. The TNF-α gene is located in IBD3.[152] Sequence variants of the promoter of the TNF-α gene include e.g. the -1031 T→ C, the -857 C→ T and the -308 G→ A polymorphisms.[153] From these the -308 G→ A SNP in the promoter region of the TNF-α gene is the most closely associated with CD.[154] Individuals homozygous for the -308 A allele produce significantly more TNF-α upon stimulation and are stronger in promoting the activity and proliferation of Th lymphocytes than individuals homozygous for the -308 G allele.[154] However, results are controversial. Other authors reported a reduced frequency of the TNF-α -308 A allele in both CD and UC patients.[155]

IL-6: Besides elevations of TNF-α, IL-6 serum levels are also higher in patients with active CD. Investigations of SNPs of the IL-6 gene behind this observation, however, did not show direct association with susceptibility to CD, although patients with the -174G/C genotype were more likely to possess ileocolonic disease than patients with a different genotype.[156] Thus some influence on disease phenotype might be observed.[156] Inflammation-mediated osteopenia frequently observed in CD patients was also not in connection with SNPs of the IL-6 gene.[157]

IL-10: The effects of TNF-α, IL-6 as well as other pro-inflammatory cytokines can be naturally suppressed by the production of IL-10. As discussed earlier, the functional deficiency of IL-10 may be an important maintenance factor for chronicity of IBD. The IL-10 gene is located on the long arm of chromosome 1.[158] Three SNPs (-1082 G→ A, -819 T→ C, -592 A→ C) in the promoter region of IL-10 and two microsatellite loci (IL10.G and IL10.R) located 1.2 kb and 4.0 kb upstream respectively have been identified.[156, 158] In Caucasians, these three SNPs constitute three haplotypes (GCC, ATA and ACC) that influence IL-10 production. Specifically, in all haplotypes, the -1082 G allele is associated with higher IL-10 production while -1082 A is connected to lower levels of IL-10 production.[156] Supporting these observations, Koss et al. found that the IL-10 -1082 A allele was associated with the down-regulation of IL-10 expression in British CD patients and controls.[159]

HSP70: Heat shock proteins (HSPs) and particularly the HSP70 family play important roles in intracellular trafficking and conformation of proteins by acting as molecular chaperons, thus being involved in immune regulation.[160] Additionally, HSPs were shown to stimulate cytokine production both as endogenous[161] and as exogenous ligands[162], partly through a CD14-dependant pathway. In humans, three members of the HSP gene, referred to as HSP70-1, HSP70-2 and HSP70-Hom has been mapped.[163] Among them, HSP70-1 and HSP70-2 encode the same protein, which is referred to as inducible HSP70. There have been reports showing the protective role of inducible HSP70 in intestinal cells.[164] HSP70 was also detected in inflamed colonic mucosa from CD patients[165], therefore the HSP70 gene family has been suggested to be a possible candidate gene in CD.[166, 167] The HSP70-2 gene has a *Pst*I polymorphic site due to an A to G transition in 1267 position of the coding region. Although the polymorphism does not cause alteration in the derived amino acid sequence, previous studies showed that a different genotype of the HSP70-2 gene was associated with a different level of mRNA expression.[168] The 1267 A→ G polymorphism was also shown to contribute to increased risk of acute renal failure in premature neonates[169] and to higher risk of severe acute pancreatitis in adults as well.[170] Moreover, two research groups have detected an association

between the 1267 A→ G SNP and increased occurrence of intestinal perforations, abscesses and fistulas in Japanese and German patients with CD.[166, 167]

1.3. AIMS

In *H. pylori* infection and in CD a Th1 dominant immune reaction can be observed. Thus, the focus of our studies was the role of the inflammatory cytokines in these disorders and possible genetic factors increasing the susceptibility to disease.

1.3.1. Specific aims of our study regarding *H. pylori* infection

►Characterization of the local and peripheral production of TNF-α, IL-6, IL-8, IL-10 and nitrotyrosine in *H. pylori*-infected and *H. pylori*–negative patients and the investigation of possible correlations between the antral tissue levels of cytokines with peripheral cytokine levels and the CagA status of the patients.

►Characterization of the putative protective effect of histamine deficiency in *H. pylori*-induced inflammation and the comparative analysis of the local cytokine responses of histamine-deficient, histidine decarboxylase knock-out (HDC KO) and wild-type (WT) mice following *H. pylori* infection.

►Investigation of the relationship between TNF-α, IL-8 and CD14 polymorphisms and the development of duodenal ulcer.

1.3.2. Specific aims of our study regarding Crohn's disease

►Investigation of the presumed roles of CD14 and IL-10 gene polymorphisms in increasing susceptibility to CD and examination of the importance of genetic variations in Hsp70-2 in influencing the clinical outcome of CD.

2. MATERIALS AND METHODS

The materials and methods of each experiment are discussed in detail in the attached manuscripts. They are only briefly referred to herein.

2.1. Experiments with human samples

2.1.1. Patients and controls

Blood was obtained by phlebotomy for serological examination and DNA purification from patients with *H. pylori* positive duodenal ulcer. From these patients gastric biopsies were taken as well. The presence of *H. pylori* was confirmed, and the severity of gastritis was graded with the Sydney Classification system.[171] DNA of patients with CD was also extracted from peripheral blood drawn by phlebotomy. In the case of gastric biopsies, specimens serving as controls were taken from *H. pylori* negative patients. Control individuals, whose blood was tested for serology and was further processed for DNA purification, were ethnically matched and randomly selected healthy blood donors. Informed consent was obtained from all patients and the projects were approved by the Clinical Ethical Committee of the Medical Faculty of the University of Szeged.

2.1.2 Cytokine assays

Biopsy specimens were homogenized and were assayed for total protein content by the Lowry method.[172] Concentrations of cytokines in sera and in biopsy homogenates were determined by ELISA assays, with the following ELISA kits: TNF-α, IL-6, IL-8 and IL-10 (BIOSOURCE). The mucosal levels of cytokines were expressed as pg/mg biopsy protein. Cytokine levels were also detected after incubation of whole blood with *H. pylori 26695*. Supernatants of whole blood cultures (WBC) were thereafter tested for the presence of the TNF-α, IL-6, and IL-8 with the previously listed ELISA kits.

2.1.3. Nitrotyrosine detection

Nitrotyrosine was detected from gastric biopsy samples by an ECL Western blot technique with the application of anti-nitrotyrosine monoclonal antibody (HM 11, HyCult Biotechnology).

2.1.4. *H. pylori* serology

The simultaneous evaluation of *H. pylori* positivity and CagA status of blood donors was executed with a Western blot assay (MICROGEN).

2.1.5. Genotyping procedures

The leukocyte DNA was isolated from peripheral blood using the High Pure PCR Template Preparation Kit (Roche) according to the kit manufacturer's instructions.

The **SNP of TNF-α (-308 G→A)** was analyzed by using PCR amplification and restriction fragment lenght polymorphism analysis (PCR-RFLP). The amplified product was digested with *Nco*I (Fermentas).

Fig. 3. PCR/ARMS analysis of the IL-8 (-251 T→A) SNP. Lanes 1. and 2. correspond to genotype AA, lanes 4. and 5. to the heterozygote genotype TA and lanes 6. and 7. to genotype TT (336 bp). Lane 3. shows a molecular weight marker. The upper band corresponds to a HLA-DRB1 gene product (796 bp) as control.

The **SNP of IL-8 (-251 T→A)** was typed by an amplification refractory mutation system (ARMS) (Fig. 3). The allele-specific primers were: 5'-CCACAATTTGGTGAATTATCA AT-3' and 5'-CCACAATTTGGTGAATTATCAAA-3'. The consensus primer was 5'-TGCCCCTT CACTCTGTTAAC-3', giving a PCR product of 336 bp.

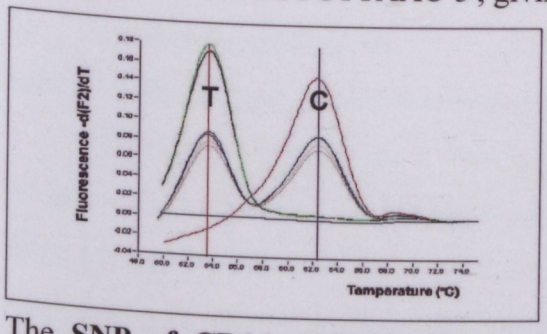


Fig. 4. Representative results of melting point analysis for CD14 (-159 C→T) SNP.
The melting point of the allele T ~53 °C (labeled as **T**) and the melting point of the allele C ~62 °C (labeled as **C**). Results of patients with homozygote genotypes show one peak while heterozygotes show two peaks at two melting points suiting the alleles.

The **SNP of CD14 (-159 C→T)** was genotyped using a rapid-cycle PCR with specific fluorescence-labeled hybridization probes on a LightCycler instrument and subsequent fluorscent probe melting point analysis (Fig. 4.).

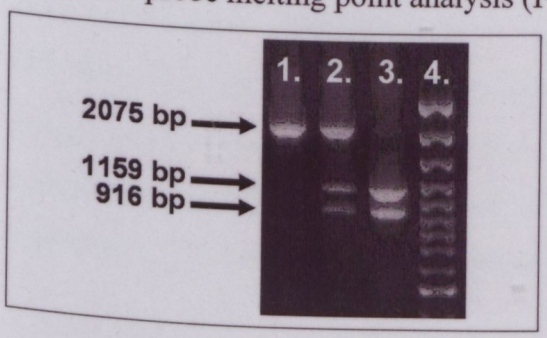


Fig. 5. PCR/RFLP analysis of the HSP70-2 (1267 A→G) SNP.
Lane 1. correspond to genotype AA (2075 bp), lane 2. to the heterozygote genotype AG (2075, 1159 and 916 bp) and lane 3. to genotype GG (1159 and 916 bp). Lane 4. shows a molecular weight marker.

The **SNP of IL-10 (-1082 G→A)** promoter polymorphism was analyzed by the PCR-RFLP method. The amplified product was digested with *Ear*I (New England Biolabs).

The **SNP of HSP70-2 (1267 A→G)** was assessed by means of a PCR-RFLP procedure. The amplified product was digested with *Pst*I (Fermentas) (Fig. 5.).

2.1.6. Statistical analysis

All statistical calculations were performed with the GraphPad Prism4 statistical program. When computing the different levels of cytokine production the Mann-Whitney, and Student tests were used. When the levels of significance for the genotype and allele frequencies were analyzed, the chi-square test with Yates' correction or the Fisher's exact test was used. The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium (HWE) by the χ^2 test, with one degree of freedom used. For comparison of age and sex between the patients and the controls in case of the *H. pylori* genetic experiments, the Mann-Whitney U test and the Fisher's exact test was used. To compare the continuous clinical data characteristic of the subgroups of CD patients and controls, one-way ANOVA was used. The Student-Newman-Keuls test was utilized for *post hoc* pairwise multiple comparisons. In all tests, a probability level of $p<0.05$ was taken as an indication of statistical significance.

2.2. Animal experiments

2.2.1. Animals and *in vivo* treatments

Histidine decarboxylase knockout mice (HDC KO) and wild type (WT) (CD1 background) mice were used. Histamine-deficient mice were prepared by using a known homologous recombination method in embryonic stem cells.[173] To avoid loading with exogenous histamine, mice were fed a histamine-free diet. *H. pylori 26695* were administered intragastrically to both HDC KO and WT mice (Fig. 7.). Parallely control groups were treated with vehicle. The ketamine-xylazine anaesthetized animals were sacrificed 8 weeks after the first infection. Blood and stomach samples were obtained individually. The

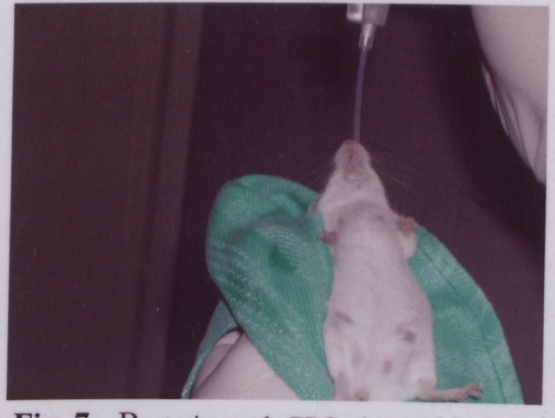


Fig. 7. Bacteria and PBS (controls) were administered intragastrically with a flexible, sterile cannula.

stomach of each mice was removed without the fundus and the sample was cut longitudinally. One quadrant of the sample was examined histologically, while the remainder was used in the ELISA and PCR experiments. The experiments were performed in accordance with the European Communities Council derivatives (86/609 ECC) and approved by the Animal Health and Food Control Station at Szeged.

2.2.2. Cytokine assays

Stomach mucosal samples were homogenized and the total protein content was determined by the Lowry method.[172] TNF-α, IL-6 and IL-10 levels in the homogenates were measured with ELISA kits (BIOSOURCE). The mucosal levels of cytokines were expressed as pg/mg of total protein content.

2.2.3. Anti-*H. pylori* IgG assays

The anti-*H. pylori* IgG levels were determined by *H. pylori* IgG ELISA (Dia.Pro.). As the ELISA kit used was initially created for human samples, the procedure was modified by using a goat anti-mouse IgG Horseradish Peroxidase Conjugate (Bio-RAD) as a secondary antibody. The analysis of the IgG antibody subclasses was carried out, focusing on mouse IgG1 and IgG2a (Serotec).

2.2.4. Bacterial strain and culture

The *H. pylori 26695* strain used was a gift from Prof. D.E. Berg (Department of Molecular Biology and Genetics, Washington University Medical School, St. Louis, USA). *H. pylori* was grown on Columbia agar (Sifin) with 5% defibrinated sheep blood and incubated in microaerophilic conditions, using gas generating sachets (bioMèrieux) at 37°C for 3 days.

2.2.5. Histopathology

Gastric sections were prepared according to a standard histological protocol and evaluated for signs of inflammation. A three grade score was used in the evaluation process: no inflammatory cells (score=0), inflammatory cells in the mucosa (score=1) or transmucosal inflammation (score=2). Three sections were examined from each stomach. Slides were processed and examined without knowledge of the experimental design or treatment group.

2.2.6. Verification of the presence of *H. pylori* in the stomach with PCR

DNA was extracted from stomach samples by a modified technique after Feng et al.[174] The 294 bp fragment of the urease C gene in *H. pylori,* encoding a required accessory protein for urease expression was detected with an oligonucleotide primer pair described by Bicley et al.[175]

2.2.7. Statistical analysis

The values obtained from the experiments on the infected and uninfected HDC KO and WT mice were compared by means of one-way ANOVA. The Student-Newman-Keuls test was used for *post hoc* pairwise multiple comparisons. In all tests, a probability level of $p<0.05$ was taken as an indication of statistical significance. All statistical calculations were performed with the GraphPad Prism4 statistical program.

3. RESULTS

3.1. Local and peripheral cytokine response and CagA status of *H. pylori* positive patients with duodenal ulcer *(Paper I.)*

3.1.1. The levels of TNF-α, IL-6, IL-8 and IL-10 in human biopsy specimens

DU patients infected with *H. pylori* exhibited a higher antral cytokine (TNF-α, IL-6, IL-8 and IL-10) production than did *H. pylori* negative patients *(Fig. 1. in Paper I.)*. There was an inverse correlation between the levels of TNF-α and the anti-inflammatory cytokine IL-10 *(Fig. 2. in Paper I.)*.

3.1.2. Mucosal nitrotyrosine detection and result of the histopathology analysis

In 23 of the 40 biopsy samples, considerable nitrotyrosine production was detected by ECL Western blotting *(Fig. 3. in Paper I.)*. Nitrotyrosine was detected in those samples, in which the highest TNF-α and IL-8 cytokine concentrations were measured. In a comparison of the nitrotyrosine assay results with the Sydney scores all of these 23 samples were evaluated at grade 3.

3.1.3. Comparison of serum cytokine levels and *ex vivo* inducible cytokine release in whole blood from DU patients and healthy blood donors

There was no difference in mean of TNF-α, IL-6 and IL-8 concentration in the sera of the DU patients and the blood donors. *H. pylori 26695* induced moderate TNF-α and IL-6 production when WBC were coincubated with *H. pylori* for 18 h. There was no significant difference between the amounts of these cytokines in supernatants of whole blood from the DU patients and healthy blood donors. The *in vitro H. pylori* infection caused a higher IL-8 production in the DU patients than in the controls *(Table 1. in Paper I.)*. DU patients exhibited a significantly higher IL-8 production at the periphery than that of the healthy blood donors. There was no further difference in

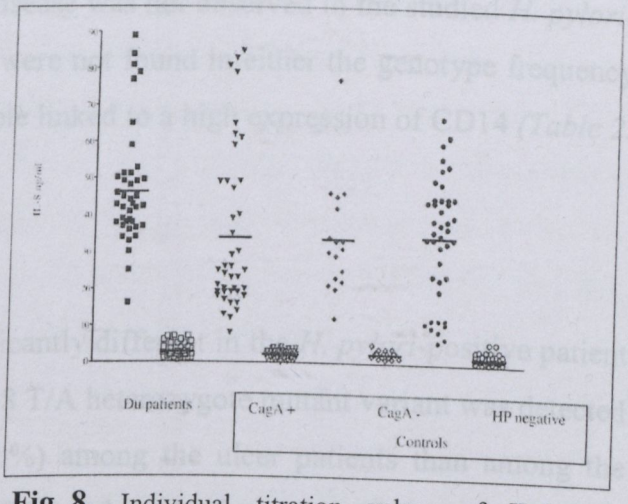


Fig. 8. Individual titration values of IL-8 in supernatants of WBCs.
WBCs stimulated with *H. pylori* (filled symbols) and not stimulated -basal level of IL-8- (open symbols).

in vitro cytokine-producing capacity between the different subgroups of controls (*H. pylori*$^+$ Cag A$^+$, *H. pylori*$^+$ Cag A$^-$, *H. pylori*$^-$) (Fig. 8.).

3.1.4. CagA status

CagA positivity was demonstrated in 39 of the 40 patients with DU, and in 41 of the 58 *H. pylori* positive healthy blood donors.

3.2. Genetic polymorphism of interleukin-8 (IL-8) is associated with *H. pylori*-induced duodenal ulcer *(Paper III.)*

The genotype frequencies of the TNF-α, CD14 and IL-8 polymorphisms in the control group did not deviate significantly from those expected for the HWE. Among the DU patients only the IL-8 genotype frequency deviated significantly from that for the HWE.

3.2.1. SNP of TNF-α (-308 G→ A)

There was no significant difference in the distribution of the TNF-α -308 G→ A gene polymorphism between the *H. pylori*-positive DU patients and the *H. pylori*-positive healthy controls. Likewise, no significant difference in the rate of carriage of the high-secreting allele was seen between the two populations *(Table 1. in Paper III.)*.

3.2.2. SNP of CD14 (-159 C→ T)

A significant correlation between the presence of the CD14 -159 C→ T promoter polymorphism and the development of DU disease was not observed in the studied *H. pylori*-positive populations. Significant differences were not found in either the genotype frequency distributions, or the rate of carriage of the allele linked to a high expression of CD14 *(Table 2. in Paper III.)*.

3.2.3. SNP of IL-8 (-251 T→ A)

The genotypic frequencies were significantly different in the *H. pylori*-positive patient and healthy control groups (Table 1.). The IL-8 T/A heterozygote mutant variant was detected with a significantly higher frequency (65.22 %) among the ulcer patients than among the healthy, *H. pylori*-positive blood donors (36.17 %), while the frequency of the normal allelic genotype (T/T) was significantly higher in the control group (44.68 % vs 15.94 %). The rate of carriage of the high-secreting allele (IL-8 A) was significantly different in the two populations: 51.45 % among the DU patients vs 37.23 % in the healthy blood donors.

	H. pylori-positive DU patients		*H. pylori*-positive controls	
	N	%	n	%
Allele				
T	67 [a]	48.55 [a]	59 [a]	62.77 [a]
A	71 [a]	51.45 [a]	35 [a]	37.23 [a]
Genotype				
T/T	11 [b]	15.94 [b]	21 [b]	44.68 [b]
T/A	45 [b]	65.22 [b]	17 [b]	36.17 [b]
A/A	13	18.84	9	19.15

[a] Fisher's exact test: p=0.043, OR=1.7863, CI 95%: 0.23-0.95.
[b] Chi-square test with Yates' correction: χ^2=11.26, p=0.0008.

Table 1. IL-8 (-251 T→ A) genotype distribution for DU patients and controls

3.3. Effects of *H. pylori* infection on gastric inflammation and local cytokine production in histamine-deficient (histidine decarboxylase knock-out) mice *(Paper II.)*

3.3.1 Local TNF-α, IL-6 and IL-10 production from the mucosal specimens of mice

H. pylori infection led to considerable cytokine responses in the gastric mucosa in the infected groups. As compared with the non-infected groups, the local TNF-α level was increased significantly in the gastric samples in both groups of animals infected with *H. pylori*. However, the TNF-α production was significantly smaller in the HDC KO animals than in the WT group (Fig. 9.A). The infection also enhanced IL-6 production in both groups. Again, the IL-6 response was significantly weaker in the HDC KO mice than in the WT animals (Fig. 9.B). In parallel with the pro-inflammatory cytokines, *H. pylori* infection significantly stimulated the local production of IL-10, an anti-inflammatory cytokine, in both experimental groups. However, the IL-10 levels in the infected HDC KO and WT mice did not differ (Fig. 9.C).

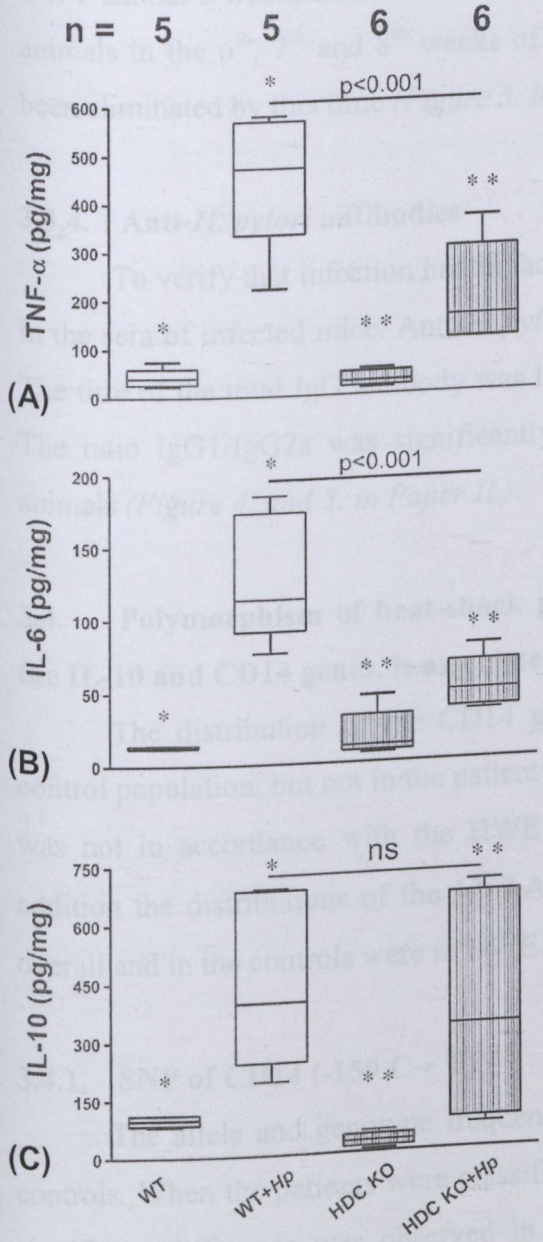


Fig. 9. Production of local cytokines (A: TNF-α, B: IL-6 and C: IL-10) in the homogenized murine mucosa, expressed as "pg/mg" of total protein content.

3.3.2. Histopathology

Morphological signs of inflammation were clearly observed in the gastric mucosa of the infected mice in both groups. When graded with respect to the involvement of the mucosa (grade 1) or the mucosa+submucosa (grade 2) in the inflammation, the inflammation proved less severe in the HDC KO mice than in the WT mice *(Figure 2. in Paper II.)*.

3.3.3. PCR directly on infected gastric tissue

H. pylori UreC specific DNA was checked for 8 weeks in the gastric samples of *H. pylori*-infected WT and HDC KO mice. Such DNA was detected in the first 4 weeks from gastric samples of both *H. pylori*-infected WT and HDC KO mice, but only from 1 sample of a WT animal 5 weeks after the infection. Specific DNA was not detected in our experimental animals in the 6^{th}, 7^{th} and 8^{th} weeks of *H. pylori* infection, suggesting that the bacteria had been eliminated by this time *(Figure 3. in Paper II.)*.

3.3.4. Anti-*H. pylori* antibodies

To verify that infection had in fact occurred, anti-*H. pylori* antibodies were determined in the sera of infected mice. Anti-*H. pylori* antibodies were detected in both infected groups. The titre of the total IgG antibody was higher in the WT animals than in the HDC KO mice. The ratio IgG1/IgG2a was significantly different only in the *H. pylori*-infected HDC KO animals *(Figure 4. and 5. in Paper II.)*.

3.4. Polymorphism of heat-shock protein gene HSP70-2, but not polymorphisms of the IL-10 and CD14 genes, is associated with the outcome of Crohn's disease *(Paper IV.)*

The distribution of the CD14 genotypes was in accordance with the HWE in the control population, but not in the patient group. While, the distribution of the IL-10 genotypes was not in accordance with the HWE in the either control or the patient population. In addition the distributions of the 1267 A→G genotypes of the Hsp70-2 gene in the patients overall and in the controls were in HWE.

3.4.1. SNP of CD14 (-159 C→ T)

The allele and genotype frequencies did not differ between the CD patients and the controls. When the patients were classified according to the clinical course of their disease a significant difference was observed in the genotype frequency of the patients with more advanced disease as compared both with the control group and with the less severe patient

group. This difference is a direct consequence of the significant differences detected in both the C/C vs. C/T and the C/T vs. T/T genotype frequency analyses *(Table 3. in Paper IV.)*.

3.4.2. SNP of IL-10 (-1082 G→ A)

There were no significant differences in the allele or genotype frequencies of the IL-10 gene between the patients and the controls. Further stratification of the patients into subgroups on the basis of the clinical data did not affect the outcome of the analysis *(Table 3. in Paper IV.)*.

3.4.3. SNP of Hsp70-2 (1267 A→G)

A significant difference in allele or genotype frequency was not observed between the patients and the controls. When the patients were classified according to the clinical course of the disease, again no significant difference was observed *(Table 3. in Paper IV.)*. Further analysis of the Hsp70-2 gene polymorphisms however led to significant differences between the non- operated and operated patient groups (Table 2.). When we compared the G/G genotype frequencies of the operated and non-operated patients (24 % vs. 8 %) with the A/A and A/G genotype frequencies (32 % vs. 37 % and 44 % vs. 55 %) of the same groups, significant differences could be found. Comparison of the relationship of the A carrier state of the patients with the frequency of surgery revealed that those patients who do not carry the A allele are at greater risk of surgery (8 % vs. 24 %).

	Operated					
	No		Yes		Control	
Hsp70-2 1267 A→G	*n*	%	*n*	%	*n*	%
AA	25	37[a]	19	32[a]	28	39
AG	37	55[b]	26	44[b]	34	48
GG	5	8[a,b]	14	24[a,b]	9	13
A carrier (GA, AA)	62	92[c]	45	76[c]	62	87
GG	5	8[c]	14	24[c]	9	13

Abbreviations: Hsp = heat-shock protein; OR = odds ratio.
[a]χ^2 test with Yates' correction, $p = 0.05$; [b]χ^2 test with Yates' correction, $p = 0.026$; [c]χ^2 test with Yates' correction, $p = 0.021$.
OR = 3.858; 95% CI = 1.29–11.49.

Table 2. Hsp70-2 (1267 A→G) genotype distribution for operated patients and controls

5. DISCUSSION

5.1. The inflammatory response following *H. pylori* infection

5.1.1. The cytokine status of *H. pylori* positive ulcer patients

The generally accepted hypothesis about the pathomechanism of *H. pylori*-related diseases attributes a central role to the overproduction of cytokines and other inflammatory substances and their consequent damaging effects. Our examination of antral biopsy specimens from Hungarian DU patients also demonstrated significantly higher levels of TNF-α, IL-6 and IL-8 compared to *H. pylori*-negative subjects. In addition, we detected nitrotyrosine, as a marker of peroxynitrite formation, in those samples in which the highest TNF-α and IL-8 cytokine concentrations were measured. Accordingly, we can conclude that these cytokines, which activate inflammatory cells, will result in the generation of nitrating and oxidizing agents. This can lead to increased rates of DNA damage in the gastric mucosa, inducing NO production and the production of oxygen free radicals, and resulting overall in peroxynitrite formation. Considerable evidence indicates that tyrosine residues in proteins become nitrated preferentially at sites of inflammation-induced tissue injury, leading to the suggestion that nitrotyrosine may be deleterious or serve as a biomarker for the effects of reactive nitrogen oxides.[57] These free radicals are of pathophysiological importance in the destruction of the gastric mucosa. The apoptotic effect of peroxynitrite is extremely important in this process.

The level of IL-10, the important counter-regulatory cytokine was also measured in gastric specimens. Despite individual variations, gastric tissues displayed relatively low levels of this cytokine. Thus, a negative correlation can be observed between the local production of IL-10 and TNF-α, indicating an imbalance in the induction of the pro-inflammatory cascade and inhibitory cytokines.

In our in vitro experiments the IL-8-inducing ability of the *H. pylori 26695* $CagA^+$ strain was higher in white blood cells originating from patients with DU. Regarding the production of the other cytokines no significant alteration was detected in the periphery. Furthermore, the *H. pylori* positive and $CagA^+$ normal blood donors (without a history of ulcer disease) exhibited the same basal *in vitro* inducible IL-8 producing capacity as that of the *H. pylori* negative controls. These observations imply, that there might be a possibility that the intensive local production of IL-8 in the gastric mucosa of patients with DU and the higher IL-8 producing ability of the peripheral white blood cells originating from DU patients

are connected. Therefore, it was tempting to speculate that genetic factors could be responsible for the elevated IL-8 response which led us to investigate the IL-8 polymorphism.

5.1.2. The effect of histamine-deficiency on *H. pylori*-induced inflammation

The effect of several novel agents has been investigated in the treatment of *H. pylori* infection. However, a few of these aimed at the abrogation of inflammation.

One of the factors which plays an essential role in numerous immune reactions is histamine. Besides its well-characterized effects in acute inflammatory and allergic responses, histamine also influences the expression and actions of various cytokines.[176] Therefore, we studied the effect of histamine-deficiency on *H. pylori*-induced inflammation. Our experiments showed a lower production of inflammatory cytokines (TNF-α and IL-6) after *H. pylori* infection in HDC KO mice compared to WT animals. This might be explained by the lack of the pro-inflammatory effect of histamine. Contrary to the TNF-α and IL-6, IL-10 responses did not reveal such a marked difference between the two experimental groups. Thus, the imbalance that can be detected between the Th1/Th2 cytokines was higher in the WT group than in HDC KO mice. This decrease in Th1 bias is an important factor against inflammation sustenance and subsequent symptom prevalence. The weak inflammatory response in the HDC KO mice is also supported by morphological data, merely a superficial involvement of the mucosa is notable.

The attenuation of the immune response in HDC KO mice following *H. pylori* infection is remarkable because the pronounced immune activation is responsible for most of the intensive mucosal damage observed during the infection in WT animals and patients. Although the total deficiency of histamine can not be achieved, it can be partially reproduced in humans by pharmaceutical methods through the usage of antihistamines. In the past, antihistamines, H_2 receptor antagonists, had been used in the eradication therapy. But the eradication rate for these medications hand-in-hand with antibiotics was lower compared to a combination of proton-pump inhibitors and antibiotics.[177] This led to the discontinuation of the use of these drugs. However, these antihistamines were used as antisecretory agents since they only acted through H_2 receptors, which affect gastric acid secretion and do not modulate cytokine production. The anti-inflammatory function would be mediated through H_1 and H_4 receptors. Thus, the introduction of H_1 receptor antagonists or the administration of the previously used non-selective antihistamines could be valuable as an accessory drug in the therapeutic palette, this way the attenuation of the cytokine response could be achieved following *H. pylori* infection.

In addition to the lower levels of TNF-α and IL-6 in *H. pylori* infected HDC KO mice, lower concentrations of anti-*H. pylori* antibodies were also detected. Besides influencing cytokine responses, it is also probable that histamine indirectly modulates immunoglobulin production through its effect on B cell activation. It has been shown that in histamine-deficient mice the inducibility of IL-6 is significantly reduced.[178] IL-6 is a pleiotropic cytokine of central importance in the generation of a systemic inflammatory reaction, B cell activation[179], etc. Thus, it may be suspected that HDC KO mice with reduced IL-6 levels will display a decreased B cell activation and consequently a lower IgG production. The specific antibody response, observed in HDC KO mice is also interesting from a therapeutic point of view. Elevated anti-*H. pylori* antibody titers have been affiliated with *H. pylori*-related extragastric diseases. A significant correlation was shown between anti-*H. pylori* titers and the degree of atherosclerosis.[11] It was demonstrated that plaques from both *H. pylori*-positive and negative patients react with anti-VacA and-CagA antibodies.[11] Thus, *H. pylori* may provoke a cross-reaction between its virulence factors and the plaques, leading to plaque instability. In addition, heat shock proteins (e.g. HSP65) released by *H. pylori* and also expressed on atherosclerotic plaques serve as antigens for anti-HSP antibodies. Anti-HSP titers were shown to correlate with increased cardiovascular risk.[11] Our group also showed an association between anti-HSP60 titers and ischaemic heart disease.[180] Furthermore, cross-mimicry between bacterial and self-antigens was shown to be a pathogenic factor in chronic urticaria patients[11] and antibodies against 19-kDa *H. pylori*-associated lipoprotein were detected in chronic urticaria.[11] Therefore, the administration of antihistamines (H_1- or non-selective receptor antagonist) might even be effective in lowering the specific antibody response associated with *H. pylori* infection. Thus, it may be valuable in the treatment of *H. pylori*-related extragastric diseases, as it has been introduced, for other reasons, into the therapeutic scheme of chronic urticaria.

5.2. The role of gene polymorphisms in the development of *H. pylori*-induced DU and Crohn's disease

In the previously listed experiments increased production of pro-inflammatory cytokines could be observed during *H. pylori*-induced inflammation. This corroborates studies from other researchers. Similar results have been published in connection with the dysregulated production of both pro-and anti-inflammatory cytokines and the dominating Th1 polarization in studies focusing on inflammation observed in CD. Numerous data suggest that

these pathogenic processes are genetically determined and it also seems likely that mutations in several genes act as etiological factors in both diseases.

5.2.1. Cytokine gene polymorphisms

Our investigation of the role of various cytokine polymorphisms towards higher cytokine production yielded different results. In *H. pylori*-positive subjects no significant connection was found between the TNF-α (-308 G→ A) SNP and the development of DU, *while a significantly higher frequency of the IL-8 -251 T/A genotype was observed among the H. pylori-positive DU patients than among H. pylori-positive, healthy subjects*. In case of the examined TNF-α (-308 G→ A) SNP the result is interesting, because of the recent findings of significant associations observed in Portuguese and Korean populations.[78,79] Thus, as an explanation of this contradiction, differences in the populations and ethnicity cannot be ruled out. However, other explanations are possible. First, it should be considered that the TNF-α gene utilizes different sets of transcriptional elements, and TNF-α protein expression is probably not regulated exclusively at the transcriptional level determined by -308 site on the promoter.[181] Furthermore, even with a potentially high TNF-producing ability, the -308 G→ A SNP may not pose a risk of ulcer development. This is in good accordance with the finding in our previous study that there was not an increased TNF-α-producing ability among DU patients in general, when their whole blood cell cultures were investigated.

The IL-8 -251 T/A genotype, which was detected in higher frequency among *H. pylori*-positive DU patients, was shown to reflect an elevated IL-8-producing ability in other studies.[182] Therefore it is not surprising, that the frequency of the TT genotype (with a relatively low IL-8-producing potential) was significantly higher among the non-DU subjects. This observation is consistent with the results of Hamajima et al.[86], who concluded that IL-8 -251 T/T individuals might display a milder inflammatory reaction following *H. pylori* infection. Among our patients, there were only a few individuals who carried the A/A genotype; it is very likely that this reflects the relatively small number of patients investigated to date. The higher incidence of the -251 T/A genotype with a concomitant higher IL-8-producing potential[182] draws attention to the importance of the genetic determination of IL-8 production in *H. pylori*-induced DU. This might verify our previous finding, that the inducible IL-8 was higher in patients with DU than in *H. pylori*-positive healthy subjects. IL-8 is a crucial cytokine in the pathogenesis of DU, and it is produced by not only inflammatory cells, but also gastric epithelial cells.[69]

Th1 polarization is crucial in both diseases. A deficiency in IL-10 production is a key factor contributing to this phenomenon in CD.[120] However, we did not observe any correlation between the examined IL-10 (-1082 G→A) SNP and CD in the Hungarian population. This is interesting in the light of the findings of Fernandez et al., who showed that the -1082 G allele, the one that supposedly causes elevation in IL-10 levels, as well as the IL10.G14 microsatellite allele were significantly increased in Spanish patients with CD.[183] The combined presence of both alleles in 1 individual notably increased the risk to develop CD.[183] Furthermore, other investigations also failed to discover any alleles which contribute to increased susceptibility to CD.[152, 156, 158, 184] Hence, we presume that this polymorphism of the IL-10 gene is not responsible for the lower IL-10 production in CD. Several reasons are possible for the failure to observe any significant association between CD and this IL-10 SNP. First, functional NOD2 is required for effective production of the anti-inflammatory cytokines IL-10 and TGF-β.[185] Patients with CD bearing the NOD2 mutation release lower amounts of IL-10, so it is very likely that the defective production of anti-inflammatory cytokines is an important feature in the pathogenic mechanism that links NOD2 mutations to CD. Second, Treg cells play a pivotal role in the control of intestinal inflammation, and both IL-10 and TGF-β are involved in their mechanism of action.[107] It is very likely that in CD there is a failure of induction of the regulatory cells, which could maintain the level of IL-10. IL-10 has a central role in the ability of Treg cells to inhibit colitis. Thus, not the IL-10 polymorphism itself, but the functional deficiency of IL-10 secretory cells could result in an imbalance of IL-10 and Th1. It is noteworthy that an insufficient function of NOD2 may be involved in the low background level of Treg activity.[186]

5.2.2. Polymorphism of the CD14 gene

The increased production of cytokines may also depend on an increased stimulus in the signal transduction pathway. Associations between genetic variations in the genes encoding microbial sensor proteins, like NOD2/CARD15, and CD have already been extensively studied and well-documented.[138-140] Polymorphisms of these genes may potentiate the effect of bacterial antigens thus shifting the production of inflammatory substances toward Th1. In our experiments we focused on the SNP (-159 C→ T) of CD14, a pattern-recognition receptor, which mainly recognizes bacterial LPS and its association with the development of *H. pylori*-induced DU and Crohn's disease. Karhukorpi et al. [144] observed a tendency to a higher frequency of the CD14 T/T genotype in DU patients as compared with

subjects without DU. Whereas, in case of CD, research findings are controversial with different results in Japanese [146] and European populations.[141, 142, 147] In our studies, however, no association between ulcer or Crohn's disease and CD14 polymorphism was evident. On one hand the discrepancies between our results and those showing a positive correlation between the examined factors may originate from the different populations involved in these studies. On the other hand further reasons are possible. Bacterial components also interact with different Toll-like receptors, mainly TLR4, when initiating the immune response therefore they should be taken into consideration as well. It is noteworthy, however, that in *in vitro* studies the gastric mucosal recognition of *H. pylori* was independent of TLR4.[187] Moreover, it was also shown that *H. pylori* is sensed rather by the intracellular sensor, NOD1 via a functional type IV secretory system.[188] In contrast, in other studies it was suggested that TLR4 may play a crucial role in the initiation of inflammatory responses to *H. pylori* infection.[189] In connection with CD earlier[190] and more recently published investigations have shown novel associations between the TLR4 (-299 A→ G) polymorphism and CD and UC.[191] Furthermore, studies in CD14 knockout mice suggest that a CD14-independent loading of LPS onto TLR4 exists, thus the significance of CD14 in the pathogenesis of *H. pylori*-induced DU and CD might be further questioned.[192, 193] But in both DU and CD further studies are warranted.

5.2.3. Polymorphism of Hsp70-2 and its significance in the severity of CD

Several gene polymorphisms have been examined by us and others, which might predispose to CD development, a few addressed protective genetic factors. The Hsp protein family has been suggested to be cytoprotective in CD and other disorders, moreover, inducible Hsp70 was shown to have a protective role in intestinal cells.[164] Therefore, we aimed to investigate the possible protective properties of Hsp70-2 in Hungarian CD patients. It has been demonstrated that the different genotypes of the Hsp70-2 gene are associated with different levels of mRNA expression; the A/A genotype has the highest level of mRNA expression, and the G/G genotype the lowest.[168] If the protective potential of the Hsp70-2 gene influences the patterns of intestinal inflammation, it is plausible that the difference in Hsp70-2 genotype could influence the outcome of the disease in our CD patients, i.e. the need for surgery. Our study suggested that the Hsp70-2 (1267 A→G) gene polymorphism is not directly associated with the susceptibility to CD, but rather with the severity of the disease. Allele A (the 'protective allele') may be associated with a less progressive form of the

disease, and therefore more frequent among CD patients who do not undergo surgery. Similar results were obtained by Esaki et al.[166] from an investigation of Japanese patients and by Debler et al. for a German population.[167] These results imply the clinical value of the genotypic assessment of the gene and also that the Hsp70-2 (1267 A→G) SNP might be of prognostic value in the severity of the disease regarding the risk of surgical intervention.

5.2.4. The significance of polymorphism studies

Association studies are emerging as a tool for understanding the genetics of common human diseases. They hold substantial promise in identifying individuals who are at greatest risk for developing certain disorders. However, several problems exist, which must be considered and corrected before appropriate conclusions can be drawn. The focus on these problems might answer why the results of investigations concerning the same topic (e.g. SNPs) are so contradictory. First, the use of genome-wide SNPs, to detect realistic effect sizes would require thousands of individuals. Therefore large scale association studies should be conducted. These should meet certain criteria set up by experts experienced in this field. Patients and controls should be chosen according to strict guidelines. Furthermore, the presence of undetected population structure, that can mimic the signal of association and lead to more false positives or to missed real effects, should be considered. The consequences of population structure on association outcomes increase markedly with sample size.[194] For the size of a study needed to detect typical genetic defects in common diseases, even the modest levels of population structure within population groups cannot be safely ignored.[194] Thus, careful consideration should precede the design and interpretation of association studies.
However, if studies are conducted appropriately, great benefits can be attained. It would become possible to target high risk individuals with selective prophylactic therapies designed to prevent/reduce the incidence of disease. In case of *H. pylori*, advantages would be best realized in countries where the prevalence of *H. pylori* infection is high. Reduced morbidity, mortality, and related health care costs could be achieved. In addition, the more selective use of antibiotics to treat only those individuals who are at highest risk would be expected to help avoid the development of antibiotic resistance.[79] Whereas, in case of patients with CD, where the exact pathogens are unknown, but certain environmental factors, e.g. the excessive intake of FODMAPs, have been identified, lifestyle changes can be recommended.[128] Patients can be advised to stop consuming FODMAPs and thus reduce the possibility of CD development or disease exacerbation. Furthermore, with the concept of "dysbiosis" as a pathogenic factor in mind, patients can be recommended to take probiotics –administration of live, beneficial

bacteria- or prebiotics –manipulation of diet to promote growth of beneficial intestinal microflora- in order to rebalance their intestinal microflora.[108] This therapy is already beginning to gain ground.[108] It is, therefore, tempting to imagine the future with children wearing a wrist band which has a digitally managed database comprising their individual SNPs, determined at birth, thus helping medical treatment if necessary.

5.2.5. The importance of Th1 dominance

Despite the well-characterized differences that can be observed in the two diseases studied in our work, important similarities can also be detected. The most significant would be the mucosal inflammation occurring as the consequence of a genetically determined, aberrant, pro-inflammatory cytokine response to constituents of the microbial flora, which in turn induces the pathological Th1 response. Thus, a common denominator in both cases is the overwhelming Th1 dominance. This raises the question, whether there is a genetically programmed inclination in both diseases towards a dominating Th1 bias.

One of the key factors, which initiates Th1 polarization is the elevated IL-12 response, this can mutually be found in *H. pylori*-induced inflammation and CD. Two susceptibility loci, one on chromosome 9 and one on chromosome 11 were identified with the help of mouse models of CD, especially the TNBS-induced colitis model.[195] The susceptibility locus on chromosome 11 is of interest as it contains, among others, the gene encoding the IL-12p40 chain. In addition, this locus is associated with susceptibility to other Th1-mediated diseases such as the experimental autoimmune encephalomyelitis (EAE), model for sclerosis multiplex (Eae6 locus) [107, 196] and the insulin-dependent diabetes in humans (Iddm18 locus).[107, 197] This indicates that this locus might contain a disease gene that is important to the Th1-cell response. Furthermore, other mouse studies of CD have also shown that another Th1-inducing cytokine, IL-23, which consists of the IL-12p40 chain linked to the IL-23p19 chain, not to the IL-12p35, as in the case of IL-12, more effectively stimulates Th1-memory-cell responses than IL-12 does.[107, 198] So it is possible that IL-23 could be more important in sustaining Th1-mediated inflammation in CD.[107, 198] In support of this possibility, IL-23p19-deficient mice, which lack IL-23 but not IL-12, are highly resistant to the development of EAE.[199]

Thus, these data draw attention to the closer investigation of the Th1 response. They also raise the question of a genetic link which might exist among diseases with Th1 dominance, insinuating the possibility of a therapeutic target.

Finally, it should also be noted that the changing epidemiology observed both in the case of *H. pylori*-related diseases and CD in the recent decades has been accompanied by similar

increases in the prevalence of several immune-mediated disorders in other organs, many of them with substantial Th1 involvement.[200] This suggests that environmental conditions associated with a modern industrialized lifestyle may be associated with alterations in immunologic development. It is possible that a modern sanitized environment with new environmental factors shifts the immune system towards responses which provide inadequate protection against pathogens, which had been well-tolerated in the past (e.g. *H. pylori*). Therefore, it is becoming more difficult to tackle the aforementioned diseases. This theory might be supported by the phenomenon called the "African enigma", which refers to the relative sparsity of *H. pylori*-related disease in Africa despite high prevalence of the bacteria. The cause might be the predominant Th2 response to *H. pylori* observed among black Africans.[25] This interesting, immune response can be induced by endemic helminth infection or may reflect a genetic predisposition selected by malaria.[25] Thus, the benign immunomodulative effect of an unsanitized environment can be observed.

5.3. SUMMARY: CONCLUSIONS AND POTENTIAL SIGNIFICANCES

The major new findings of our experiments are as follows:

► Significantly increased mucosal production of TNF-α, IL-6, IL-8 and nitrotyrosisne could be observed in antral biopsy samples in *H. pylori*-infected patients compared to *H. pylori*-negative subjects. There was a negative correlation between TNF-α and IL-10 concentrations. These findings suggest that besides the bacterial virulence factor, the host response with increased levels of pro-inflammatory cytokines and reactive oxgen and nitrogen species could be relevant to the gastric pathophysiology in *H. pylori*-induced DU.

► Cytokine overproduction is not generalized in DU patients however a significant increase in *in vitro* IL-8 production might be of importance in the pathogenesis of DU.

► *H. pylori* infection induces lower TNF-α and IL-6 secretion in the gastric mucosa in HDC KO mice than in WT animals, while IL-10 levels are similar. The imbalance between Th1/Th2 is less pronounced in HDC KO mice. In addition anti- *H. pylori* antibodies were also detected in lower concentrations in HDC KO mice. These results indicate the importance of histamine in *H. pylori*-related gastritis.

► The IL-8 (-251 T→ A) polymorphism is associated with increased susceptibility to *H. pylori*-induced duodenal ulcer disease, while no significant correlation could be revealed in case of the TNF-α and CD14 promoter polymorphisms in the examined Hungarian population. These observations correlate with our previous results of higher

IL-8 levels in gastric samples and in *H. pylori*-induced white blood cells of DU patients than in controls. Thus, attention is drawn to the possible importance of IL-8 polymorphism as a genetic predisposing factor in the pathomechanism of *H. pylori*-induced DU.

► In case of experiments connected with CD no correlation could be found between the CD14, IL-10 polymorphisms and the development or the progression of Crohn's disease in the Hungarian population. However, allele A of the Hsp70-2 gene may be associated with a less severe form of CD. This suggests a clinical value of genotype assessment in CD patients.

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